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Chromatographic identification of phenolic compounds in human urine following oral administration of the herbal medicines *Daisaiko-to* and *Shosaiko-to*

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Abstract

Chemical identification of the compounds in human urine following administration of the traditional Chinese medicines, *Daisaiko-to* and *Shosaiko-to* (*Dachaihu-tang* and *Xiaochaihu-tang* in Chinese, respectively), was achieved by using a linear relationship between the logarithm of the capacity factor, log k', and that of the volume fraction of CH_3CN, log X_s^{vol}, in the aqueous mobile phase: $-\log k' = A + B \log X_s^{vol}$. Comparison of the slope, B, and the intercept, A, between the urinary compound and its suspected authentic specimen gave satisfactory results in the chemical identification. We applied this method to the initial stage of pharmacokinetic studies on the herbal medicines and identified seven flavonoids and two anthraquinone derivatives in the urine specimens obtained after herbal administration.

Keywords: *Diasaiko-to*; *Shosaiko-to*; Flavonoids; Anthraquinone derivatives

1. Introduction

Recent interest in pharmacokinetic investigations of traditional herbal medicines compelled us to develop a general procedure for the chemical identification of compounds detected in human biofluids following herbal administration [1]. The value of the capacity factor, k', in high-performance liquid chromatography (HPLC) is a commonly used parameter in the chemical identification of compounds that are difficult to isolate from a limited volume of biofluid. However, organic chemists realize that chemical identification cannot be achieved by solely using a particular type of chromatography, such as HPLC, thin-layer chromatography (TLC), etc. The use of several types of chromatography or the use of a change in solvent type has been recommended as an approach to the problem [2].

In our present study, we evaluated an identification procedure using an HPLC apparatus equipped with a multi-wavelength UV–Vis detector, which allowed us to obtain chromatographic parameters and UV–Vis spectra simultaneously. Furthermore, we evaluated values of k' by the use of a change in the organic solvent fraction in an aqueous mobile phase solvent. There are two equations that express the thermodynamic and/or empirical relationship be-

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tween k' and the composition of the mobile-phase solvent. One of the equations was proposed by Soczewinski and Golkiewicz [3–8],

$$\log k' = c - n \log X_s^{mole} \quad (1)$$

where X_s^{mole} refers to the mole fraction of the polar solvent in the mobile phase mixtures, and c and n are constants. Later, Eq. (1) was reexamined in normal phase chromatography using free energy known tautomers [9]. The other equation proposed by Snyder and co-workers [10,11] is used in reversed-phase liquid chromatography.

$$\log k' = \log k_w - S\Phi^{vol} \quad (2)$$

Here k_w is an extrapolated value of k' in pure water, S is a constant, and Φ^{vol} is the volume fraction of the organic modifier in the mobile-phase mixtures.

We applied the above relationship to the chemical identification of nine phenolic compounds, which were newly detected in human urine following oral administration of the traditional Chinese medicines, *Daisaiko-to* (TJ-8, Tsumura, Tokyo, Japan) and *Shosaiko-to* (TJ-9). These medicines are used in Japan for the treatment of hyperlipidemia [12] and chronic hepatitis [13], respectively. As a result, we observed that the following equation was more reliable and accurate for our chemical identification.

$$-\log k' = A + B \log X_s^{vol} \quad (3)$$

Here X_s^{vol} is the volume fraction of CH_3CN in the aqueous mobile phase mixtures, and A and B are constants.

2. Experimental

2.1. Chemicals and biochemicals

The authentic specimens of rhein and aloe-emodin were obtained commercially from Extrasynthese (Genay, France), 4′,5,7-trihydroxyflavanone (naringenin) was from Aldrich (Milwaukee, WI, USA), baicalein and wogonin were from Wako (Osaka, Japan) and oroxylin-A was from Tsumura (Tokyo, Japan). Liquiritigenin was extracted and isolated from commercial licorice, as described in Ref. [14]. Medicarpin was kindly provided by Prof. Nomura of Toho University (Chiba, Japan). Davidigenin was synthesized by the method of Miura et al. [15].

Diatomaceous earth granules (particle size of 50–100 μm) were prepared from Celite No. 545 (Johns Manville, Denver, CO, USA), as described in Ref. [16]. The organic solvents and the other chemical reagents used in our study were of analytical grade and were purchased from Wako.

β-D-Glucuronidase (EC 3.2.1.31, from bovine liver) was supplied by Sigma (St. Louis, MO, USA) and had 624 000 units/g of solid. A 10-mg amount of the β-D-glucuronidase was dissolved in 1 ml of the 0.1 *M* acetate buffer (pH 4.7) to prepare an enzyme preparation with 6240 units/ml.

2.2. Herbal materials

Daisaiko-to (TJ-8) and *Shosaiko-to* (TJ-9), purchased from Tsumura, are the extract granule preparations for ethical use. Their herbal ingredients are shown in Table 1. Each ingredient herb of TJ-8 and

Table 1
Ingredients of *Daisaiko-to* (TJ-8) and *Shosaiko-to* (TJ-9)

Herbal ingredients	TJ-8		TJ-9	
	Parts	% (w/w)	Parts	% (w/w)
Radix bupleuri (Umbelliferae)	6.0	26.1	7.0	29.2
Radix scutellariae (Labiatae)	3.0	13.0	3.0	12.5
Rhizoma pinelliae (Araceae)	4.0	17.4	5.0	20.8
Rhizoma zingiberis (Zingiberaceae)	1.0	4.3	1.0	4.2
Fructrus zizyphi (Rhamnaceae)	3.0	13.0	3.0	12.5
Rhizoma rhei (Polygonaceae)	1.0	4.3		
Fructus aurantii immaturus (Rutaceae)	2.0	8.7		
Radix paeoniae (Paeoniaceae)	3.0	13.0		
Radix ginseng (Araliaceae)			3.0	12.5
Radix glycyrrhizae (Leguminosae)			2.0	8.3

TJ-9 was obtained from Uchida Wakanyaku (Tokyo, Japan). They were the dry root of *Bupleurum falcatum* Linne (*Umbelliferae*), the dry root of *Scutellaria baicalensis* Georgi (*Labiatae*), the dry tuber of *Pinellia ternata* Breitenbach (*Araceae*), the dry rhizoma of *Zingiber officinale* Rosco (*Zingiberaceae*), the dry ripe fruit of *Zizyphus jujuba* Miller var. *inermis* Rehder (*Rhamnaceae*), the dry rhizoma of *Rheum palmatum* L. & *R. tanguticum* M. (*Polygonaceae*), the dry immature fruit of *Citrus natsudaidai* Hayata (*Rutaceae*), the dry root of *Paeonia lactiflora* Pallas (*Paeoniaceae*), the dry root of *Panax ginseng* C.A. Meyer (*Araliaceae*) and the dry root of *Glycyrrhiza glabra* Linne (*Leguminosae*).

For analytical purposes, 20 mg of TJ-8 and TJ-9 were mixed with 6 ml of 0.1 *M* acetate buffer (pH 4.7) by ultrasonication for 5 min at room temperature. After standing for 30 min, the mixtures were centrifuged at 1550 *g* for 10 min. The supernatant solutions were stored at −20°C until β-D-glucuronidase treatment. The ingredient herbs were mechanically crushed to a 100-mesh powder and the aqueous components were extracted by undergoing the same procedure as mentioned above to prepare ingredient herbal extracts.

2.3. Collection of urine samples

Three healthy male volunteers, aged 24–29 years and weighing 60–71 kg, were recruited from this university campus to take part in the testing program. The subjects read and completed informed consent statements and the study was approved by the ethics committee of our faculty.

The subjects abstained from solid food and liquids, except for water, from midnight of each study day, but they received lunch 3 h after being dosed in the morning. They did not take any other medicines, alcoholic beverages, tea or coffee, or food and beverages containing ingredient herbs or their generic plants, for at least two weeks before participating in the study. A 7.5-g TJ-8 dose was orally administered to one of the subjects, while the other two took 5.0 g doses of TJ-8. Timed urine samples were collected at 0, 1, 3, 6, 9, 12, 18, 24, 30, 36 and 48 h. Following a two-week washout period, the above subjects took TJ-9 to give timed urine samples using the same procedure as that used for TJ-8.

2.4. Treatment with β-D-glucuronidase and rapid flow fractionation

The herbal solutions and urine samples were then treated with β-D-glucuronidase as follows. A 30-μl volume of the enzyme solution (187.2 units) was added to 3-ml samples that had been adjusted to a pH value of between 4 and 5 with acetic acid. The resulting mixtures were incubated at 37°C for 12 h and then stored at −20°C.

The glucuronidase-treated samples were subjected to rapid flow fractionation (RFF) before HPLC analysis. The details of this sample clean-up procedure have already been described in our previous reports [16,17]. Briefly, the RFF is a pH-dependent liquid–liquid extraction on diatomaceous earth cartridges for sample treatment. The chemical components in the enzyme-treated urine were separated into three fractions, i.e. a neutral fraction (N-f), a weakly acidic fraction (W-f) and a strongly acidic fraction (S-f).

2.5. HPLC analysis and recognition of the compounds of herbal origin

The HPLC system consisted of a solvent delivery pump (BIP-I, Jasco, Tokyo, Japan), a syringe loading sample injector (Model 7125, Rheodyne, Cotati, CA, USA), an ODS analytical column (UG120, 250×4.6 mm I.D. with 5 μm Capcell Pak C_{18}; Shiseido, Tokyo, Japan) maintained at 30°C in a column oven, and a UV–Vis multi-wavelength detector (MD-910, Jasco) that can scan between 195 and 650 nm. The signal from the detector was collected and analyzed with a DP-L910/V system (Version 7, Jasco).

The mobile phase was a mixture of H_2O–CH_3CN (67:33, v/v) containing 0.5 m*M* H_3PO_4 at a flow-rate of 1.5 ml/min. We analysed the extracts of the TJ-8 and TJ-9 herbal medicines, the extracts of the ingredient herbs, and the urine samples collected before and after administration of TJ-8 and TJ-9. The detection wavelength was set in the range of 195–400 nm, with a data acquisition time of 30 min.

In the chromatograms of the urine samples after administration of TJ-8 or TJ-9, peaks which were considered to be derived from the herbal compounds should (a) not be observed in the urine before administration, (b) be observed in TJ-8 or TJ-9, as

well as in some ingredient herbs and (c) exhibit pharmacokinetic profiles in the timed urine samples.

2.6. Isolation of urinary herbal compounds for identification

The assigned urinary herbal compounds were isolated from the urine samples that showed the highest HPLC peaks of the compounds. The mobile phase mixtures used for isolation were H_2O–CH_3CN–acetic acid (66:32:2 or 63:35:2, v/v). The eluents containing the compounds of interest were collected and evaporated to dryness under reduced pressure at 40°C. Repeated chromatographic runs under the same conditions gave satisfactory amounts of urinary compounds for the following chromatographic identification.

2.7. Chromatographic identification of urinary herbal compounds

First, we compared the peaks of urinary herbal compounds with those of TJ-8, TJ-9 and their ingredient herbal extracts in terms of chromatographic retention behaviour and UV spectra obtained using the data processing system. Second, we speculated about their tentative structures by referring to the relevant phytochemical compounds that have been described in the literature and are available commercially or elsewhere, as described in Section 2.1.

In our final stage of identification, chromatographic runs using the urinary herbal compounds and their suspected authentic specimens were performed to give the linear relationships described in Eq. (3). Three binary mobile phases were chosen for each

Table 2
Chromatographic identification of urinary compounds after oral administration of *Daisaiko-to* (TJ-8) and *Shosaiko-to* (TJ-9)

No.	Mobile phase[a]	Specimen	$-\log k' = A + B \log X_s^{vol}$		
			B [CV(%)]	A [CV(%)]	$\lvert r\rvert$[b]
1	b, c, d	Urinary **D1** from TJ-8	3.88±0.01 (0.26)	1.182±0.003 (0.25)	0.9993±0.0002
		Authentic naringenin	3.88±0.02 (0.52)	1.182±0.010 (0.85)	0.9993±0.0000
2	d, e, f	Urinary **D2** from TJ-8	3.51±0.01 (0.28)	0.698±0.005 (0.72)	1.0000±0.0000
		Authentic aloe-emodin	3.50±0.02 (0.57)	0.695±0.007 (1.01)	1.0000±0.0000
3	d, e, f	Urinary **D3** from TJ-8	3.95±0.00 (0.00)	0.777±0.001 (0.13)	1.0000±0.0000
		Authentic rhein	3.96±0.01 (0.25)	0.778±0.004 (0.51)	1.0000±0.0000
4	a, b, c	Urinary **S1** from TJ-9	3.50±0.01 (0.29)	1.258±0.004 (0.32)	0.9999±0.0001
		Authentic liquiritigenin	3.51±0.01 (0.28)	1.259±0.004 (0.32)	1.0000±0.0000
5	c, d, e	Urinary **S2** from TJ-9	4.03±0.01 (0.25)	1.110±0.003 (0.27)	0.9999±0.0001
		Authentic davidigenin	4.02±0.02 (0.50)	1.107±0.007 (0.63)	1.0000±0.0001
6	d, e, f	Urinary **S3** from TJ-9	4.16±0.00 (0.00)	0.851±0.000 (0.00)	0.9999±0.0001
		Authentic medicarpin	4.16±0.01 (0.24)	0.854±0.003 (0.35)	0.9999±0.0001
7	b, c, d	Urinary **DS1** from TJ-8	4.10±0.02 (0.49)	1.194±0.007 (0.59)	0.9988±0.0001
		Urinary **DS1** from TJ-9	4.11±0.02 (0.49)	1.198±0.007 (0.58)	0.9987±0.0001
		Authentic baicalein	4.11±0.02 (0.49)	1.199±0.007 (0.58)	0.9989±0.0001
8	d, e, f	Urinary **DS2** from TJ-8	4.25±0.03 (0.49)	0.873±0.009 (1.03)	0.9981±0.0003
		Urinary **DS2** from TJ-9	4.24±0.02 (0.47)	0.871±0.006 (0.69)	0.9982±0.0002
		Authentic wogonin	4.25±0.02 (0.47)	0.874±0.006 (0.69)	0.9981±0.0002
9	e, f, g	Urinary **DS3** from TJ-8	3.90±0.01 (0.26)	0.689±0.002 (0.29)	1.0000±0.0000
		Urinary **DS3** from TJ-9	3.90±0.01 (0.26)	0.689±0.003 (0.44)	1.0000±0.0000
		Authentic oroxylin-A	3.90±0.01 (0.26)	0.690±0.003 (0.44)	1.0000±0.0000

[a] The mobile phases (a–g) were mixtures of CH_3CN and H_2O containing 0.5 m*M* H_3PO_4. The ratios of CH_3CN–H_2O (v/v) were as follows: a, 28:72 ($X_s^{vol}=0.28$); b, 32:68 ($X_s^{vol}=0.32$); c, 36:64 ($X_s^{vol}=0.36$); d, 40:60 ($X_s^{vol}=0.40$); e, 44:56 ($X_s^{vol}=0.44$); f, 48:52 ($X_s^{vol}=0.48$); and g, 52:48 ($X_s^{vol}=0.52$).

[b] All linear relationships were statistically significant ($p<0.05$).

urinary compound and the corresponding authentic specimens. Four chromatographic runs were repeated for each of the three mobile phases listed in Table 2. The capacity factor, k', was defined by the equation, $k' = (t_R - t_0)/t_0$, where t_0 is the retention time of the unretained compound, which was calculated as 1.38 min. X_s^{vol} was defined by the volume fraction of acetonitrile in the binary mobile phase. When the solution of Eq. (3) for a urinary compound and its suspected authentic specimen are in good agreement with each other using least squares calculations of both slope, B, and intercept, A, we concluded that the two specimens were identical.

3. Results

Fig. 1 shows typical chromatograms of urine samples obtained after oral administration of TJ-8 (chromatograms 1–3) and TJ-9 (chromatograms 4–6) to one of the subjects. The blackened peaks appeared only after administration and exhibited a pharmacokinetic rise and fall of excretion rates (data not shown). Among these peaks, **D1**, **D2** and **D3** were derived from TJ-8 and **S1**, **S2** and **S3** were from TJ-9, whereas **DS1**, **DS2** and **DS3** were from both TJ-8 and TJ-9. UV spectra of these nine peaks are shown in Fig. 2. We examined the chromatograms of the herbal medicines and the ingredient herbs (data not shown) to search for peaks that showed the same retention time and UV-spectra as those of the urine specimens. We suspected that the assigned peaks in Fig. 1 correspond to the known compounds listed in Table 2.

Identifications of the urine specimens with suspected authentic compounds were achieved using Eq. (3). The results are shown in Table 2. All of the capacity factors were within an appropriate range, i.e. from 1.98 to 6.92, under our optimized chromatographic conditions. All absolute values of the correlation coefficient, $|r|$, of linearity were greater than 0.997 ($n=3$) and were statistically significant ($p<0.05$). This means that the obtained values of slope, B, and intercept, A, in Eq. (3) were satisfac-

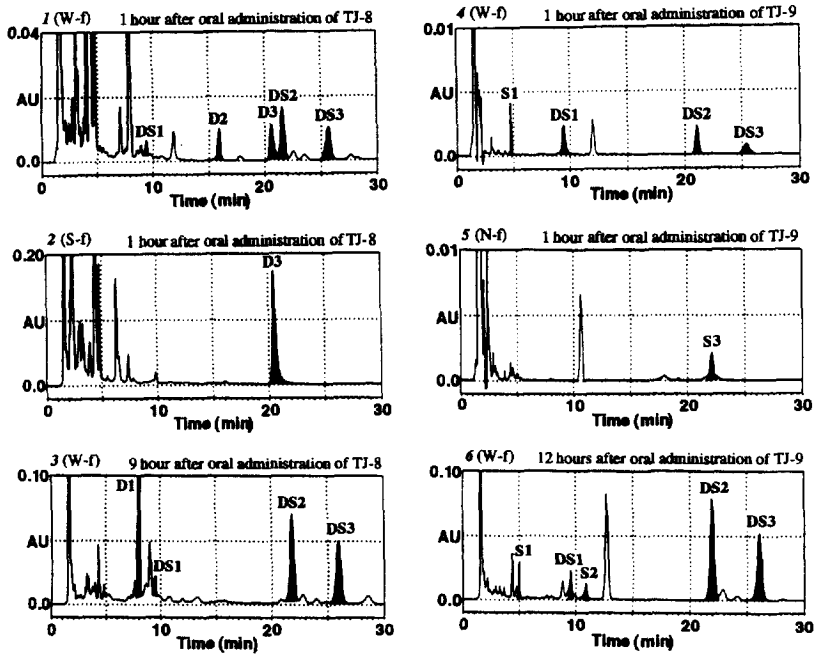

Fig. 1. Typical chromatograms of the fractionated urine samples after oral administration of *Daisaiko-to* (TJ-8) and *Shosaiko-to* (TJ-9) to one of the subjects. W-f, weakly acidic fraction; S-f, strongly acidic fraction and N-f, neutral fraction (see Section 2.4 and Ref. [17]). ODS column at 30°C; H_2O–CH_3CN (67:33, v/v, containing 0.5 m*M* H_3PO_4) at a flow-rate of 1.5 ml/min; UV detection from 195 nm to 400 nm (for plotting above chromatograms, 225–400 nm). The peak **D1**, observed in Chromatogram 3, did not appear in Chromatogram 1.

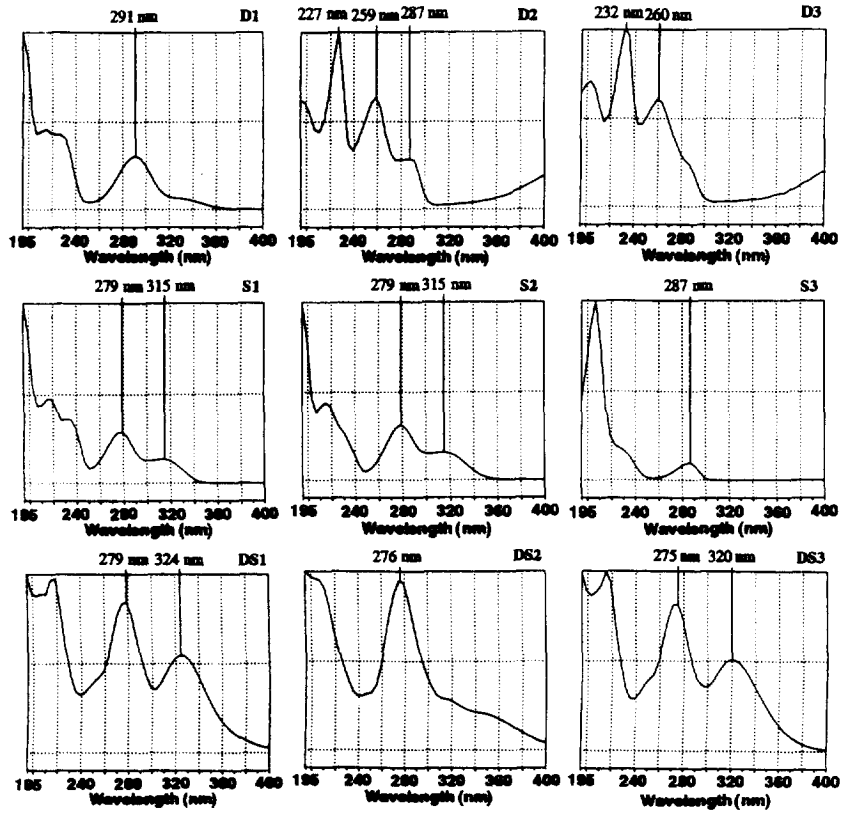

Fig. 2. UV spectra of urinary compounds derived from *Daisaiko-to* (TJ-8) and *Shosaiko-to* (TJ-9).

torily reliable to be used for the identifications. In Table 2, there were no statistically significant differences in the slope and intercept between the urinary compounds and their authentic specimens. Meanwhile, the coefficients of variation, CVs (%), of the slope and the intercept were small, suggesting high precision in our measurements. Fig. 3 shows a graphic view of the equations obtained from the urinary compounds. The graph of the authentic specimens was completely superimposed on Fig. 3 (data not shown). Thus, the herbal compounds detected in human urine following oral administration of TJ-8 or TJ-9 were identified with their authentic specimens, as described in Table 2 and also in Fig. 4.

The t_R values of rhein (**D3**), medicarpin (**S3**) and wogonin (**DS2**) were very close, so it seemed difficult to differentiate these compounds from each other. However, these compounds had statistically significant differences in their A and B values, as shown in Table 2 ($p<0.001$).

4. Discussion

Chemical identification of a sample in a very small quantity is the first barrier in pharmacokinetic studies of herbal medicines. Many organic chemists emphasize that the conventional method of sample identification by the use of a particular chromatographic retention system is untrustworthy. Various empirical and theoretical considerations have been focused on both normal and reversed-phase HPLC in terms of the linear relationship between the value of k' and the mobile-phase solvent composition [3–8,10,11,18], however, the results are still controversial. We reexamined the relationship using our

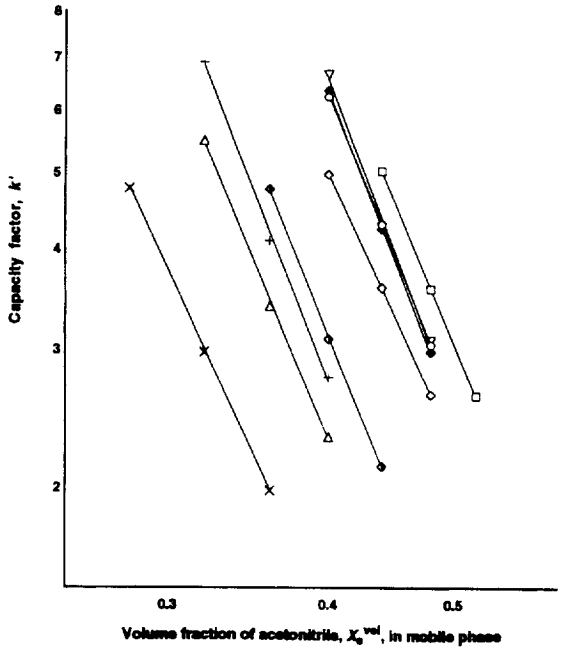

Fig. 3. Observed linear relationship between the concentration of acetonitrile and the capacity factor. △, Naringenin (**D1**); ◇, Aloe-emodin (**D2**); ○, Rhein (**D3**); ×, Liquiritigenin (**S1**); ◆, Davidigenin (**S2**); ◈, Medicarpin (**S3**); +, Baicalein (**DS1**); ▽, Wogonin (**DS2**); □, Oroxylin-A (**DS3**).

present data and found that better linearities could be expressed using Eq. (3).

As described in the preceding section, we were able to identify nine phenolic compounds of herbal origin in human urine following oral administration

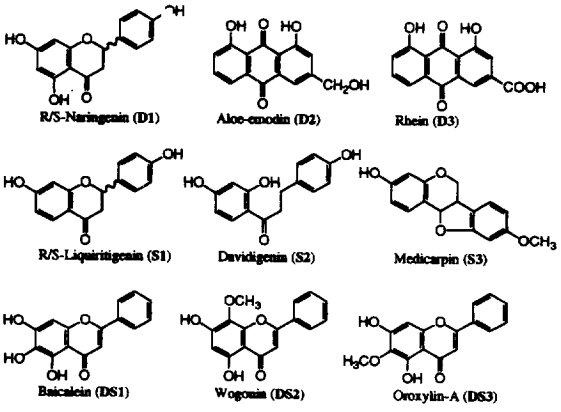

Fig. 4. Chemical structures of urinary compounds derived from *Daisaiko-to* (TJ-8) and *Shosaiko-to* (TJ-9).

of TJ-8 or TJ-9, both of which are traditional Chinese medicines, widely used in Japan under the national health insurance system. Seven of these phenolic compounds were flavonoids and the other two were anthraquinone derivatives. Although the traditional Chinese medicines have been used in China and Japan for over 1000 years, pharmacological estimations are still controversial. Two major clinical questions about the medicines are (a) what chemical substances are actually absorbed into the body and how do they achieve their pharmacokinetic profiles and (b) what are the biological effects of the absorbed substances and what roles do they play in the pharmacodynamic involvement of their clinical effects? To solve these questions, it is important to identify the compounds of herbal origin in human biofluids at low concentrations.

Many physicians and pharmacognostic chemists suspect that major components of the ingredient herbs are absorbed into the body. According to our results, shown in Fig. 4, the compounds detected in urine were not only major components but minor metabolites as well. This suggested that the chemical constitution in traditional medicines might not be equal, qualitatively and quantitatively, to those in the body after administration. Our method, described in this report, is sensitive and reliable enough to be used in chemical identification prior to pharmacological investigation of traditional herbal medicines that have been used in Japan, China and other countries for the treatment of chronic human diseases for over a millennium.

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